Modular assembly of functional DNA-based systems

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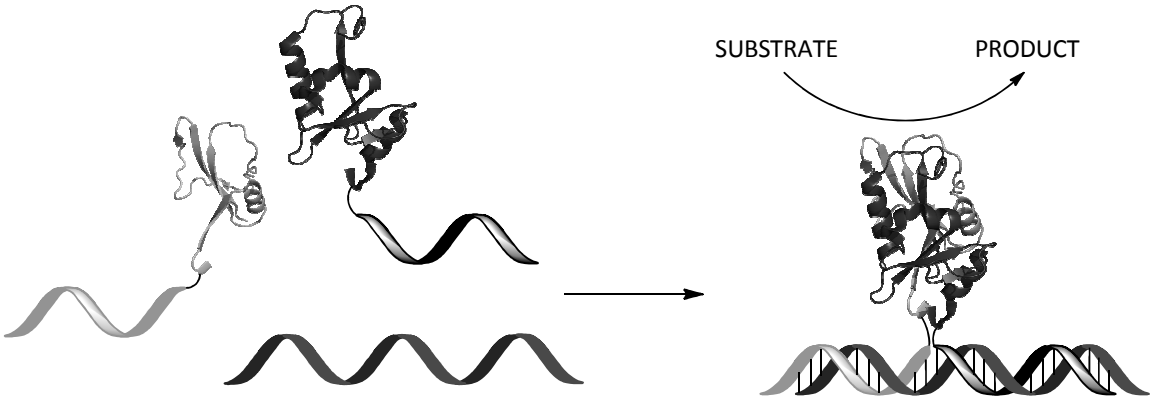
Chapter 4

Control over enzymatic activity by DNA-directed split enzyme reassembly

In this chapter the control over the activity of split murine dihydrofolate reductase (mDHFR) by DNA hybridization is investigated. The approach involves the use of protein fragments appended to oligonucleotides complementary to a DNA template. The synthesis and purification of protein fragment-oligonucleotide conjugates is described. The results show that the activity of the enzyme is dependent on the number of mismatches in the DNA template as well as on its concentration.



Parts of this chapter have been published:

N. Sancho Oltra, J. Bos, G. Roelfes, *ChemBioChem* **2010**, *11*, 2255-2258

4.1. Introduction

4.1.1. *Allostery*

Metabolic pathways are tightly regulated to control the concentration of metabolites in the cell. While a number of different methods are used to achieve such regulation, perhaps the most fascinating is control of enzyme activity through allostery, in which binding of an effector molecule either increases or decreases the catalytic activity (Figure 1).[1, 2] Thus, the activity of allosteric enzymes is modulated by the chemical composition of their environment.

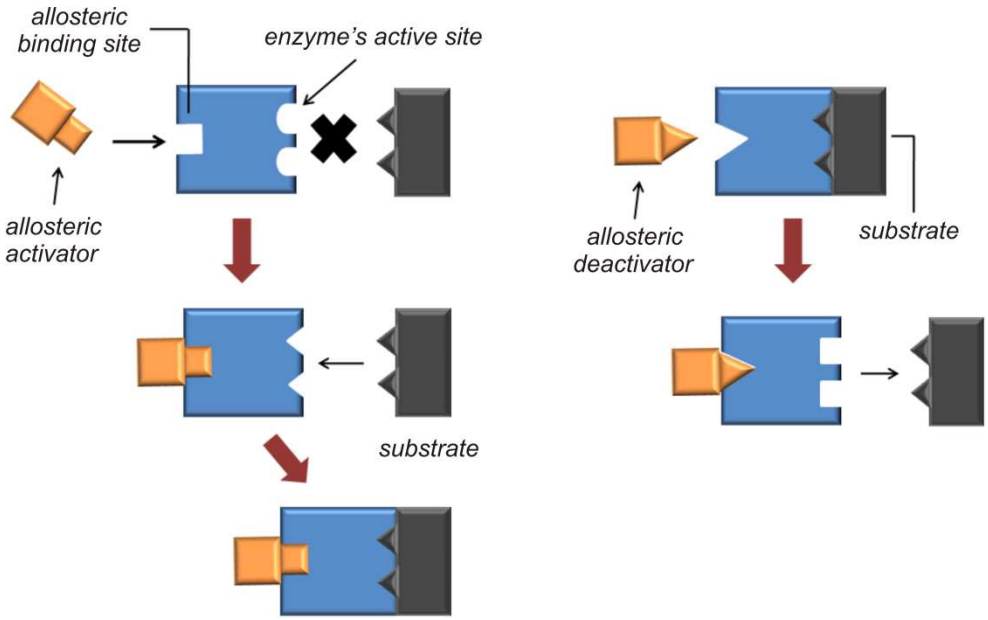


Figure 1. Schematic representation of control over enzyme activity through allostery.

In recent years, attempts have been made to design such effects into existing non-allosteric enzymes,[3] which is potentially interesting for applications such as chemical sensors, since this provides a mechanism for signal amplification.[4] A potentially versatile approach to designed allosteric enzymes involves introduction of non-proteinogenic moieties in an enzyme, which can be used to control enzyme activity.[5] For this purpose, DNA is particularly attractive; the highly specific basepairing interactions can be used to modulate enzyme activity with an exceptional degree of control.[6] A few approaches in which DNA hybridization has been used to control enzymatic activity have been reported to date; these include mechanical induction of conformational changes in enzymes or peptidic ligands,[7-9] rigidification of a DNA tether between an enzyme and a competitive inhibitor[10] and directed assembly of multi-enzyme systems.[11, 12] In this chapter, a novel approach to DNA-controlled enzymatic activity involving a split enzyme system that can be reassembled into a catalytically active conformation via hybridization of the protein-fragment-conjugated oligonucleotides to a template DNA strand is studied.

4.1.2. *Split proteins*

Split proteins have emerged as versatile and attractive tools for in vivo and in vitro sensing applications for protein-protein interactions and protein-DNA and -small molecule binding.[13-19] Recently, split proteins have been combined with nonproteinogenic recognition moieties,[20] including DNA oligomers.[21, 22] The assembly of the recognition moieties results in the folding of the split protein in an active conformation that would not be possible without the presence and recognition of these modules. To date different approaches have been followed but these have been limited to the functional, non-templated, recombination of fluorescent and luminescent proteins (Figure 2). Moreover, the only example reported of split-protein reassembly by oligonucleotide recognition[22] (Figure 2b), before the present work, makes use of unpurified and poorly characterized material. A similar approach has been reported for a split peroxidase DNAzyme.[23, 24]

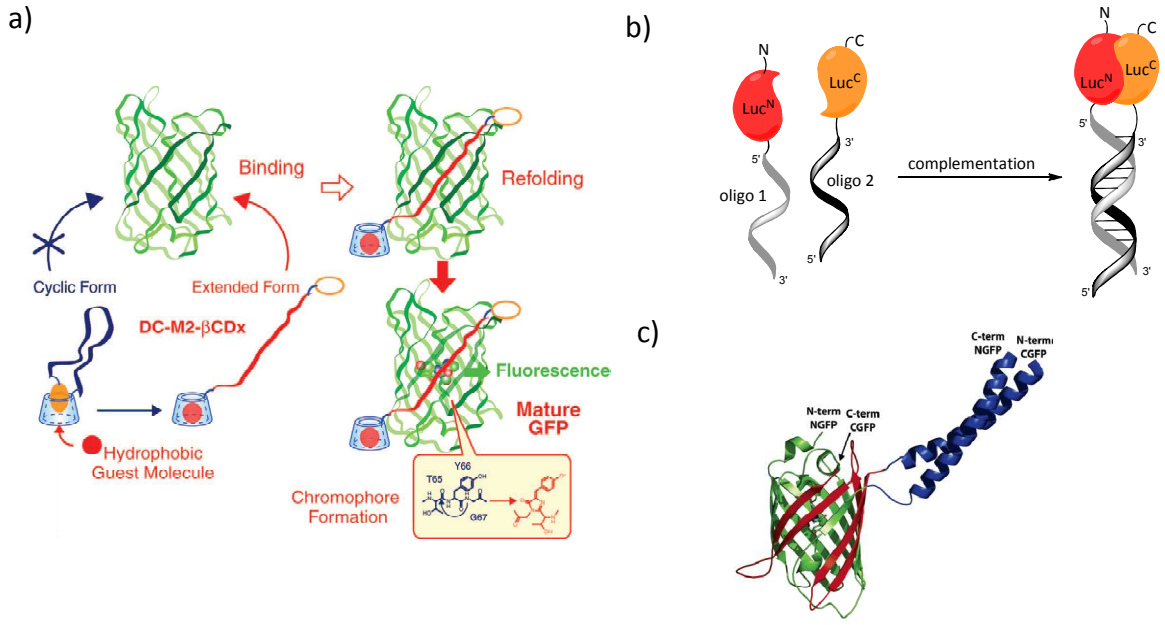


Figure 2. Split-protein reassembly by a) β-cyclodextrin – coumarin,[20] b) oligonucleotide[22] and c) protein[25] recognition. Figure panels a) and c) are reproduced with permission from [20] and [25], respectively.

In this chapter, an allosteric enzymatic system based on a split enzyme equipped with oligonucleotides conjugated to both protein segments is studied; upon binding of a complementary external DNA strand the enzyme is re-assembled in an active conformation, allowing catalysis to occur (Figure 3).

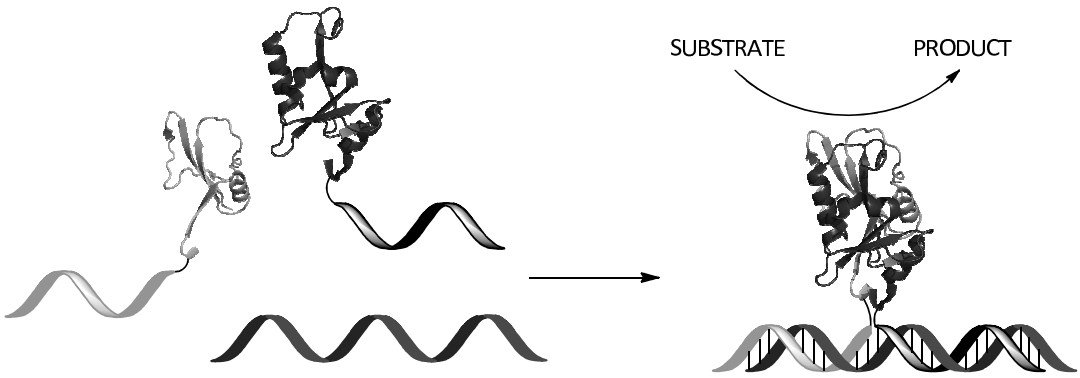
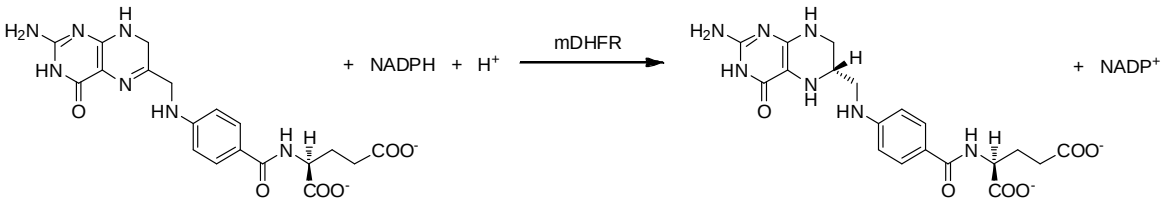


Figure 3. Schematic representation of split-enzyme activation by DNA hybridization.

This novel approach will be applied in the detection of mismatches in the DNA template sequence. Since enzymes are able to perform multiple turnovers, the application of the system as a very sensitive biosensor can be envisioned.

4.2. mDHFR fragments: cloning and expression

The design was based on the enzyme *murine* dihydrofolate reductase (mDHFR), which catalyzes the NADPH dependent reduction of dihydrofolate to tetrahydrofolate (Scheme 1).



Scheme 1. NADPH dependent reduction of dihydrofolate to tetrahydrofolate catalyzed by mDHFR.

mDHFR was selected because it can be dissected into two fragments, which can be recombined to give a stable protein, as has been demonstrated for this enzyme and the homologous *E. coli* enzyme. However, a secondary covalent or non-covalent interaction between the two protein segments is required for catalytic activity to be restored.[18, 26-29] Based on these studies, mDHFR was disconnected between amino acids 105 and 106 on the genetic level.

A maleimide coupling strategy was selected for the preparation of the mDHFR fragment – oligonucleotide conjugates. This strategy is well established for the coupling of peptides and oligonucleotides[30] and requires the mDHFR fragments to be equipped with a nucleophilic cysteine residue since mDHFR does not possess cysteine residues in its structure. The two fragments of mDHFR, i.e., N_{term}-mDHFR, corresponding to residues 1-105 and C_{term}-mDHFR, corresponding to the 106-186 fragment, were cloned and expressed independently. A C-terminal cysteine was added on the genetic level to N_{term}-

mDHFR along with an N-terminal 6xHis tag for purification purposes (Figure 4). In the case of the C_{term}-mDHFR fragment the incorporation of a terminal cysteine was investigated following different approaches. The first approach involved the incorporation of a protease (Tobacco Etch Virus (TEV) protease) recognition site (ENLYFQ-C) on the N-terminus to give C_{term}-mDHFR (1) (Figure 4). This recognition site contained a cysteine immediately after the glutamine residue at the scission site. Although the most commonly used recognition site contains a glycine instead of a cysteine, the latter sequence has been proven to be recognized by the TEV protease as well.[31] After expression, the protein fragment can be digested by proteolysis resulting in an N-terminal cysteine.

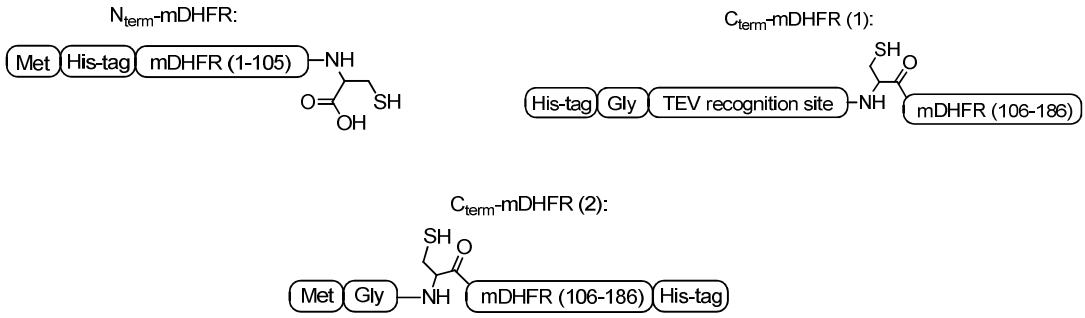


Figure 4. Schematic representation of the composition of N_{term}-mDHFR and C_{term}-mDHFR (1) and C_{term}-mDHFR (2).

The expression of the fragment was successfully conducted and the mass of the protein fragment was confirmed by ESI. However, problems arose during the TEV proteolysis. Due to the low solubility of the protein fragment the proteolysis had to be performed in the presence of urea. The minimum concentration of urea required to maintain the protein in solution was found to be 2 M. Additionally, NaCl was added to a final concentration of 150 mM to stabilize the native form of the protease. At this urea concentration the protease was still active. Nevertheless poor conversions towards the cleaved product were obtained (Figure 5). Possibly, the TEV site was not accessible to the protease due to the formation of secondary structures under the proteolysis conditions.

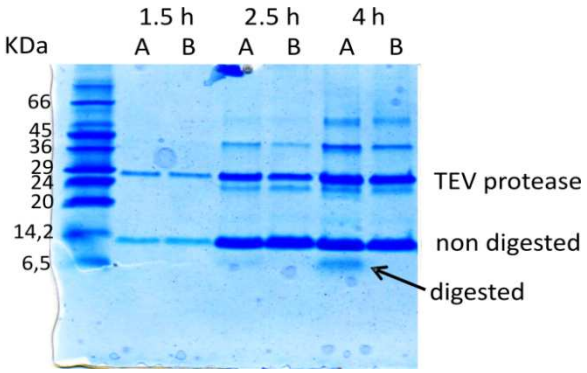


Figure 5. 12% polyacrylamide SDS-Tricine gel of the proteolysis of C_{term}-mDHFR (1) followed in time in A) 0.2 M and B) 2 M urea solution. 20 U of protease were added after each sample.

The second approach followed involved the incorporation of a cysteine at an internal position. The cysteine needed for conjugation was included to the N-terminus as part of an MGC extension to give C_{term}-mDHFR (2). A glycine residue was introduced between the start amino acid (methionine) and the cysteine to reduce the steric hindrance provided by the first amino acid that could interfere in the maleimide coupling. Additionally, a C-terminal 6xHis tag was included (Figure 4).

The mDHFR fragments were expressed in *E. coli* BL21 (DE3) and purified under denaturing conditions on a Ni-agarose column and analyzed by gel electrophoresis (Figure 6) and ESI mass spectrometry. Expression yields of 11 mg/L and 29 mg/L were obtained for N_{term}-mDHFR and C_{term}-mDHFR (2), respectively. In the case of the C_{term}-mDHFR fragment a mass corresponding to the loss of the N-terminal methionine was found; m/z = 10694 Da (calcd. M-Met: 10692 Da). This is often observed in the expression of proteins. In both cases the dimer was formed as well, as confirmed by ESI.

a) b)

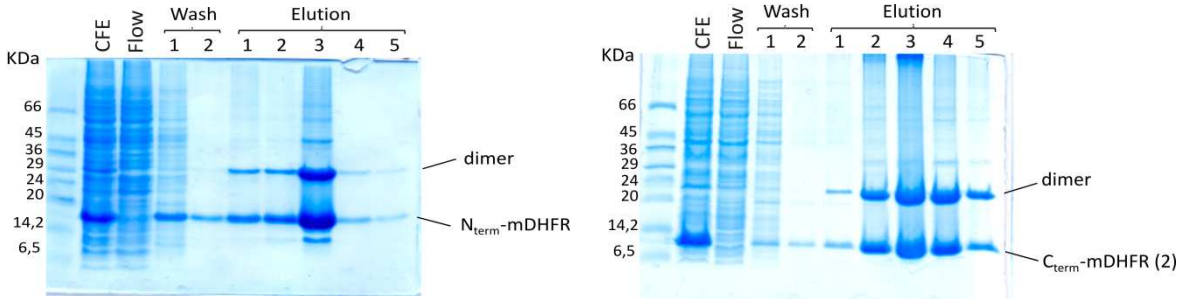
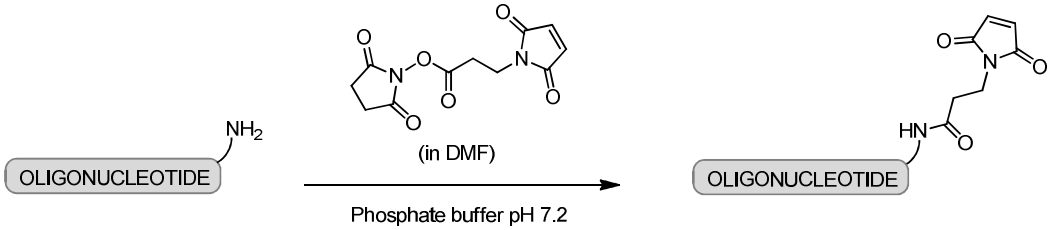


Figure 6. 12% polyacrylamide SDS-Tricine gels of the purification by Ni-NTA of a) N_{term}-mDHFR and b) C_{term}-mDHFR (2).

4.3. Maleimide modification of oligonucleotides

16-Mer oligonucleotides were used in the conjugation to the mDHFR fragments, as these provide a strong thermodynamic driving force for the split enzyme reassembly upon hybridization with the template DNA. The maleimido 5' and 3' modified oligonucleotides were prepared by treatment of the corresponding terminal amino-modified oligonucleotides with 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (Scheme 2). The optimal reaction time was found to be 2 hours since longer reaction times resulted in the hydrolysis of the maleimide group. The resulting maleimide functionalized oligonucleotides were purified by size exclusion chromatography and analyzed by reversed phase HPLC and MALDI-TOF. Isolated yields were typically around 90% as determined by UV measurements at 260 nm.

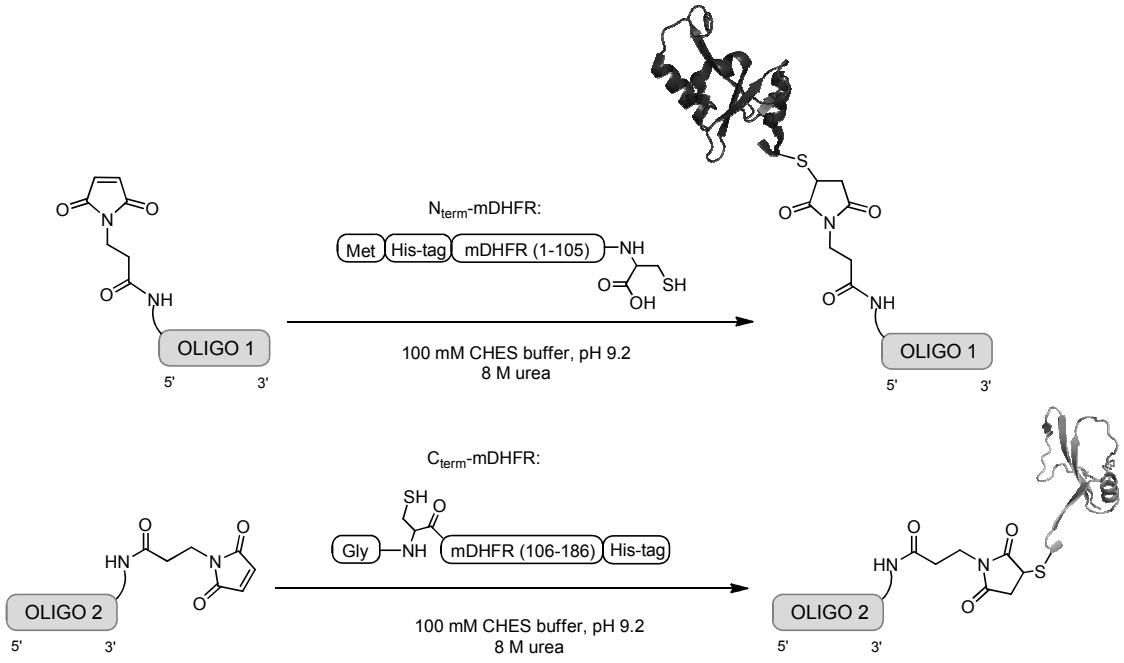


Scheme 2. Maleimide functionalization of amino modified oligonucleotides.

4.4. Synthesis and purification of protein-DNA conjugates

4.4.1. *Synthesis via maleimido coupling*

The conjugation of N_{term}-mDHFR and C_{term}-mDHFR fragments to 5' and 3' oligonucleotides, respectively, was achieved by a maleimide coupling (Scheme 3) under denaturing conditions, to prevent the precipitation of the protein fragments.



Scheme 3. General coupling procedure between maleimide functionalized oligonucleotides and mDHFR fragments. Oligo 1 is: (2-aminoethyl-6-hexylcarbamate) -5'- d(GACATGTCTGACCTTG) – 3' and Oligo 2 is: 5' d(GACTGGTGAGAACGCT) - 3'- aminohexyl.

The pH turned to be of major importance. A pH of 9.2 was chosen because at this pH the thiol group of the cysteine is predominantly in its thiolate form (pKa = 8.33) which is a more reactive species. However, the protein fragments were obtained in phosphate buffer pH 4 as eluted form the Ni-NTA column thus a rebuffering step was required. Attempts to rebuffer to 100 mM CHES (pH 9.2), 8 M urea using a desalting column was

time-consuming and resulted in the dimerization of the fragments and therefore no coupling to the oligonucleotides occurred. A faster alternative involved the precipitation of the protein with 25% TFA solution followed by decanting, washing and redissolving it in the desired buffer. This approach was applied in the coupling of N_{term}-mDHFR to Oligo 1, resulting in a conversion to the product around 10% but, unfortunately, the dimer formation could still be observed (Figure 7). In order to prevent the dimer formation, reducing agents, i.e., TCEP and $NaBH_4$, were added to the reaction mixture but similar results were obtained.

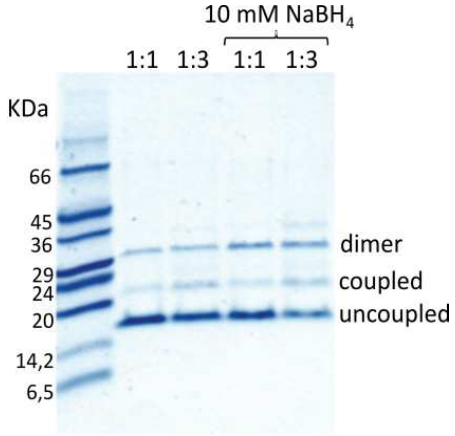


Figure 7. 12% polyacrylamide SDS-Tris Tricine gels of the reaction mixture of N_{term}-mDHFR and Oligo 1 at rations 1:1 and 1:3 in the absence and the presence of $NaBH_4$.

Dimerization could be prevented by treatment of the protein fragments with DTT prior to the coupling in order to ensure the presence of the protein in its monomer form followed by precipitation with 25% TFA, redissolving in CHES buffer pH 9.2, 8 M urea and quick addition to the freeze-dryed maleimide modified oligonucleotide. Furthermore, the coupling could be promoted by performing the reaction at 40 ^{o}C. Conversions were estimated from PAGE gel to be around 70% for the N_{term}-mDHFR fragment and around 10% for the C_{term}-mDHFR (2) fragment (Figure 8).

a) b)

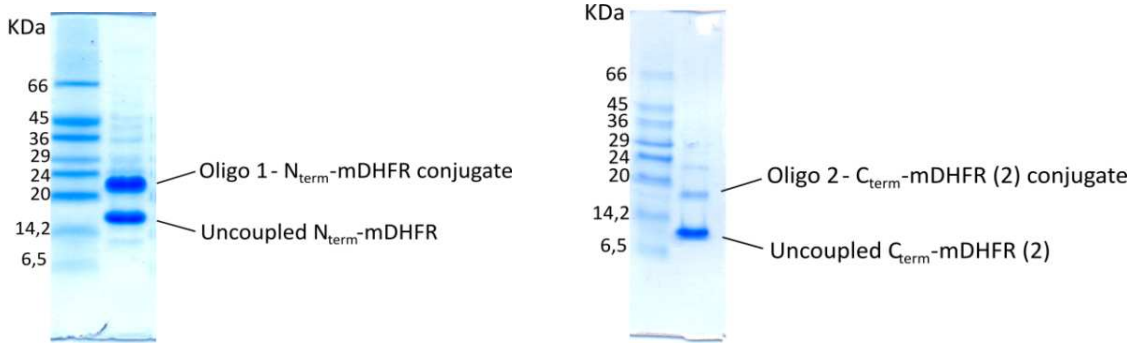


Figure 8. 12% polyacrylamide SDS-Tris Tricine gels of the reaction mixture of a) Oligo 1 + N_{term}-mDHFR and b) Oligo 2 + C_{term}-mDHFR (2).

4.4.2. *Optimization of the coupling between Oligo 2 and C_{term}-mDHFR*

The bulkiness of the DNA and the protein fragment was initially thought to be a possible cause for the low conversion obtained to Oligo 2-C_{term}-mDHFR (2). The steric hindrance could influence the accessibility of the maleimide group and the internal cysteine. Therefore, increasing the distance between the reactive groups and the DNA was envisioned as a possible solution since their availability for reaction would be in this way higher. The linker used in Oligo 1-N_{term}-mDHFR was slightly longer and therefore, the maleimide group was possibly better available for reaction. Hence, oligonucleotides functionalized with a longer linker were synthesized as previously described using in this case 6-maleimidohexanoic acid. Unfortunately, the conversion obtained when coupling the 3' modified oligonucleotide to C_{term}-mDHFR (2) was comparable to that obtained previously.

The secondary structures adopted by the single stranded DNA in solution could possibly affect the coupling as well. These different conformational structures could depend on the DNA sequence. Hence, couplings to oligo's containing different sequences were investigated. Once again, this resulted in no improvement of the coupling.

The low conversion could also be attributed to the steric hindrance provided by the amino acid residues surrounding the reactive cysteine. Therefore, a flexible linker containing amino acids GGSGG was introduced on the genetic level between the cysteine and the start of the mDHFR fragment giving C_{term}-mDHFR (3) (Figure 9).

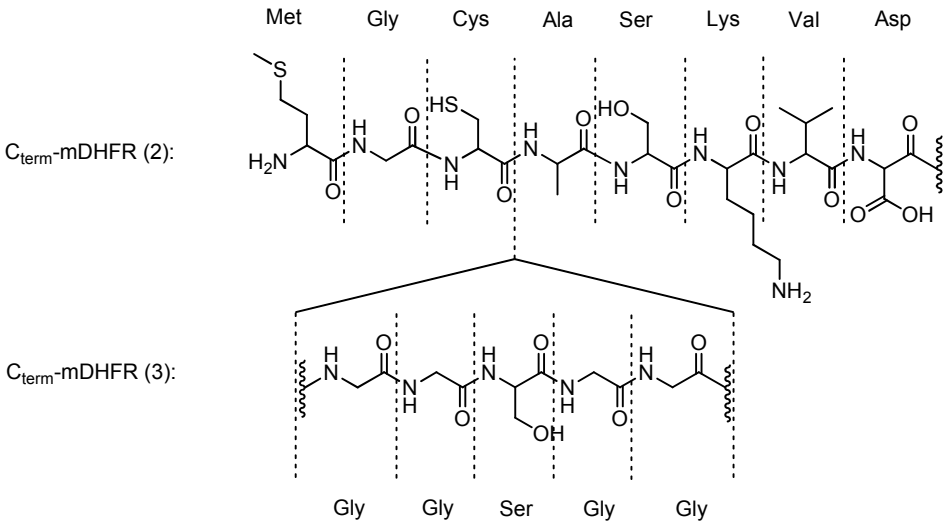


Figure 9. Initial amino acid sequence of C_{term}-mDHFR (2) and C_{term}-mDHFR (3).

The protein fragment was expressed, purified and analyzed analogous to C_{term}-mDHFR (2) obtaining an expression yield of 5 mg/mL (Figure 10).

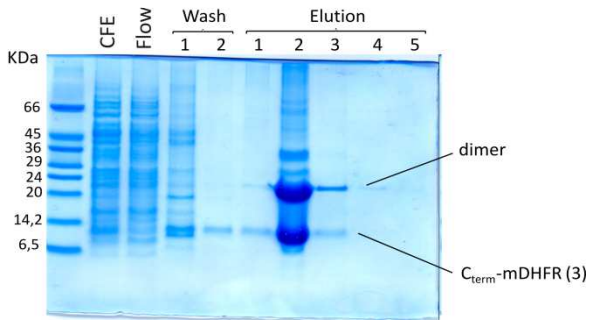


Figure 10. 12% polyacrylamide SDS-Tris-Tricine gel of the purification by Ni-NTA of C_{term}-mDHFR (3).

The conjugation reaction between 3' maleimido modified oligonucleotide and C_{term}-mDHFR (3) proceeded in this case with a considerably higher conversion, that is, around 40% (Figure 11). This confirms that the steric hindrance provided by the amino acids surrounding the reactive cysteine is important for the effectiveness of the coupling.

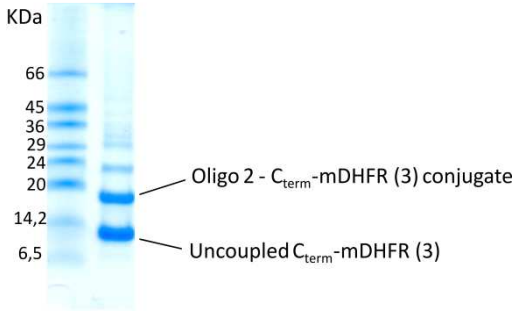


Figure 11. 12% polyacrylamide SDS-Tris Tricine gel of the reaction mixture of Oligo 2 + C_{term}-mDHFR (3).

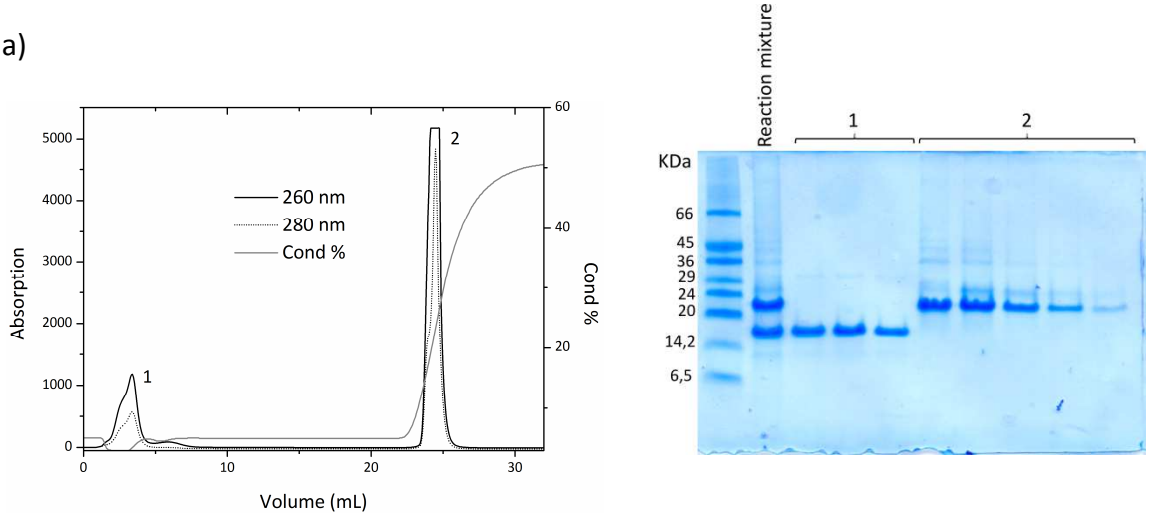
Due to the nature of the conjugates, a positively charged protein attached to a negatively charged oligonucleotide, difficulties in the purification step could be anticipated. In order to simplify the purification the use of an excess of either protein or oligonucleotide to promote the total consumption of one of the macromolecules was investigated. Ratios of oligonucleotide to protein higher than 1:1 resulted in multiple products corresponding to the non specific coupling of the oligonucleotide to different residues in the protein. However, when an excess of protein was used (up to 4 equivalents) the non specific coupling was not observed but dimer formation occurred and the conversion towards the conjugated product was comparable to when using 1:1 ratio. Moreover, size exclusion chromatography analysis showed that uncoupled oligonucleotide remained present in the reaction mixture. This implied that the purification from free protein and free oligonucleotide was necessary in this case, also.

4.4.3. *Purification of protein-DNA conjugates*

Since the coupling did not reach full conversion in any of the cases, the protein-oligonucleotide conjugates required the purification from both the uncoupled protein and the uncoupled oligonucleotide.

Purification techniques like size exclusion chromatography, preparative gel electrophoresis and the use of centrifugal filters proved unsuccessful. The overall negative charge of the conjugates allowed for purification from uncoupled mDHFR by anion exchange chromatography on a HiTrap QFF column analogous to published procedures[32] (Figure 12). Using this technique, the positively charged uncoupled protein did not get retained in the column at pH 7.5 and could be easily separated from the conjugates and uncoupled DNA.

a)



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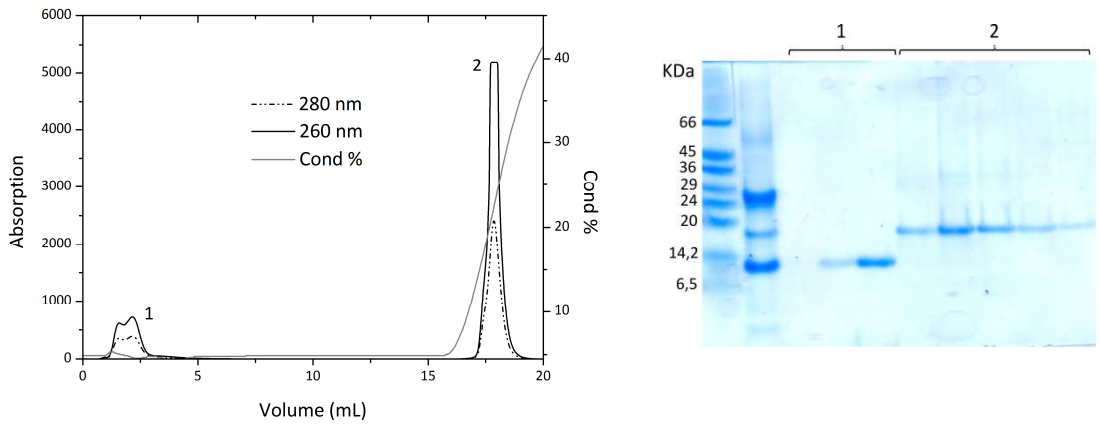
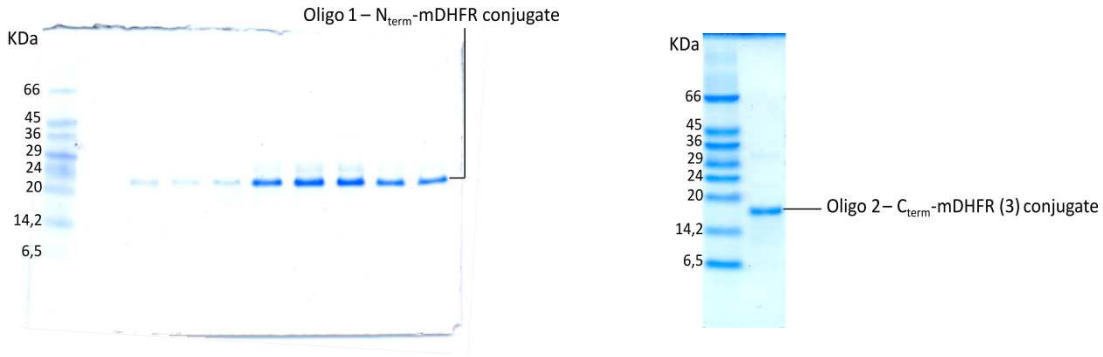


Figure 12. Purification of oligo-protein conjugates from uncoupled protein via anion-exchange chromatography. UV traces of the elution and 12% polyacrylamide SDS-Tris Tricine gels of eluted fractions for the coupling of a) Oligo 1 with N_{term}-mDHFR and b) Oligo 2 with C_{term}-mDHFR (3). 1 corresponds to the uncoupled mDHFR and 2 corresponds to the oligo-protein conjugate.

Purification from uncoupled oligonucleotide was achieved by affinity chromatography on a Ni-NTA column, taking advantage of the 6xHis tags on the protein fragments. Moreover, this last step also results in concentration of the protein-DNA conjugates. This concentration step is necessary since the reassembly of the system in the catalysis experiments will be done via rapid dilution in the reaction buffer. This purification

procedure gave rise to highly pure protein DNA conjugates, as was confirmed by PAGE (Figure 13a) and analytical size exclusion chromatography (Figure 13b), which demonstrated that the material was free of unconjugated DNA (unconjugated DNA elutes at 12 mL).

a)



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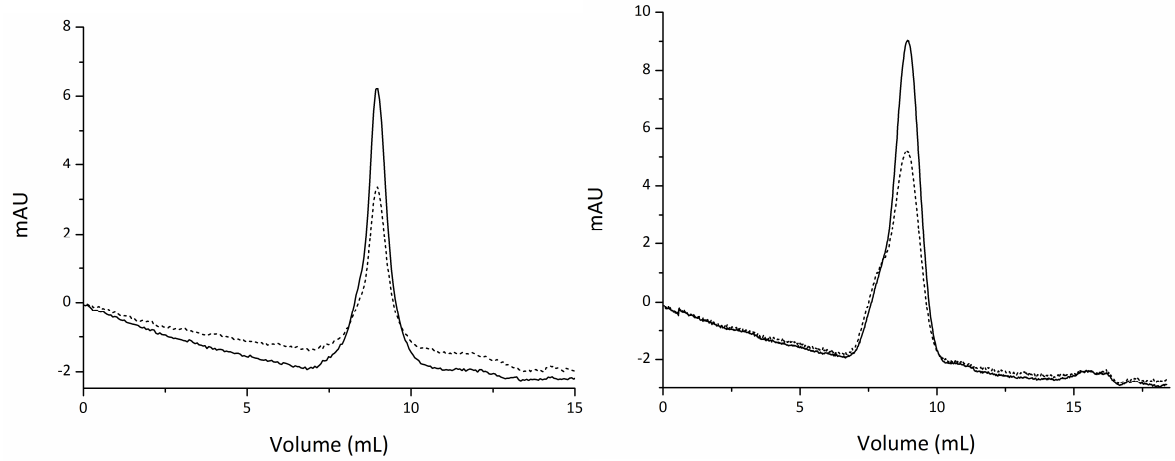
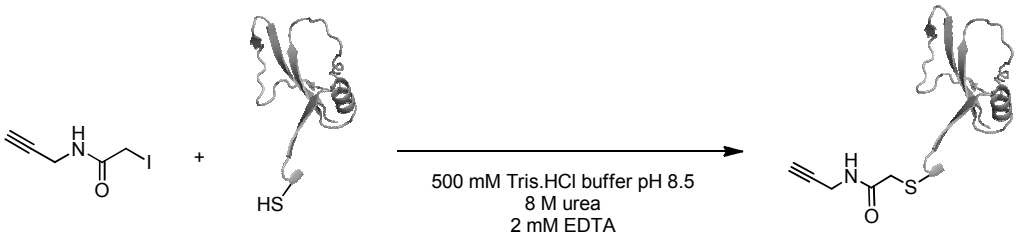


Figure 13. a) 12% polyacrylamide gels after Ni-NTA column and b) analytical size exclusion chromatography traces of pure Oligo 1-N_{term}-mDHFR (left) and Oligo 2-C_{term}-mDHFR (3) (right) conjugates. (—) corresponds to 260 nm and (·····) corresponds to 280 nm.

4.4.4. *Synthesis via 1, 3 – dipolar cycloaddition*

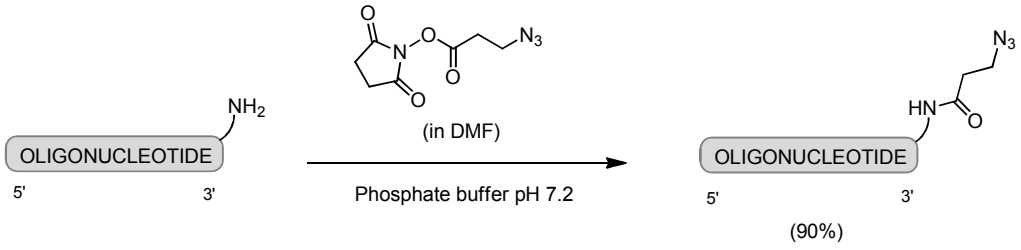
Although the maleimide strategy turned to be a convenient way to couple the oligonucleotides to the protein fragments, other alternatives were investigated in an attempt to improve the conversion of the coupling of C_{term}-mDHFR to 3'modified oligonucleotides. The coupling reaction studied was the Cu(I) catalyzed 1, 3 -dipolar cycloaddition between an azide and a terminal alkyne which is known to proceed effectively in aqueous solutions for the ligation of peptides to oligonucleotides.[33] This strategy required the functionalization of the protein fragment with an alkyne (Scheme 4)

which was achieved by reaction of the protein fragment with 2-iodo-N-(prop-2yn-1yl)acetamide. MALDI-TOF analysis of the product confirmed the presence of only the modified protein fragment. Hence, purification steps were not required.



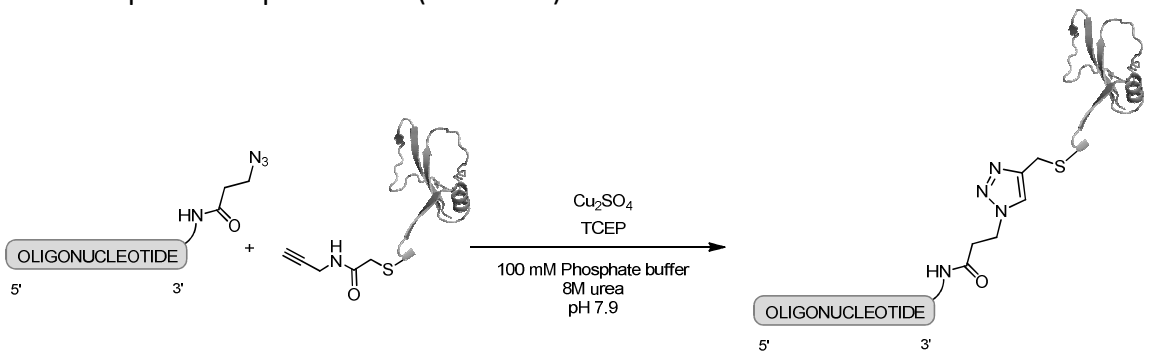
Scheme 4. Alkyne functionalization of C_{term}-DHFR (2).

The oligonucleotide was equipped with an azide group by reacting 3' amino functionalized oligonucleotides with the activated ester of 3-azidopropanoic acid (Scheme 5).



Scheme 5. Azide functionalization of amino modified oligonucleotides.

The protein fragment was conjugated to the oligonucleotide in the presence of a copper (II) salt and a reducing agent (tris(2-carboxyethyl)phosphine (TCEP)) in urea solution based on published procedures (Scheme 6). [33]



Scheme 6. 1, 3-dipolar cycloaddition between 3'modified oligonucleotides and C_{term}-mDHFR.

Different ratios and concentrations of DNA, $CuSO_4$ and TCEP were tested. The highest conversion obtained was around 70% when 1500 equivalents of $CuSO_4$ were used (Figure 14).

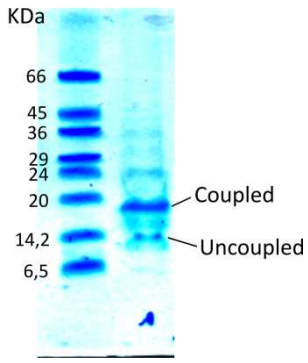


Figure 14. 12% polyacrylamide SDS-Tris Tricine gel of the reaction mixture of 1, 3-dipolar cycloaddition of 3' azide- functionalized oligonucleotide and alkyne functionalized C_{term}-mDHFR (2).

Unfortunately, the purification of the conjugate turned to be very troublesome. The difficulties in purification were attributed to the presence of a large excess of copper. A blue precipitate appeared after the reaction most likely corresponding to $Cu_3(PO_4)_2$ and which also contained the coupled product. The precipitate could be easily redissolved by addition of EDTA which coordinates the copper ions. However the conjugate showed no retention in the Ni-NTA column during the purification. Possibly, the 6xHis tag present in the protein fragment remained blocked by the copper ions since it is a coordinative site with high affinity for metals. The presence of EDTA represented an additional complication in the purification process. At the pH at which the purification from free protein is pursued (pH 7.5), EDTA is present in its deprotonated form and is retained in the anion exchange column. Subsequently, the EDTA elutes together with the product and, once loaded on the Ni-NTA column coordinates to the Ni^{2+} ions preventing the retention of the conjugate. Due to the difficulties encountered in the purification procedure, this approach was not pursued further and the study was conducted using the pure conjugates obtained via the maleimide coupling strategy.

4.5. Split mDHFR reassembly by DNA hybridization

The catalytic activity of the system was evaluated in the NADPH dependent reduction of dihydrofolate to tetrahydrofolate. The standard DHFR assay was used[34] which involves the monitoring of the consumption of NADPH by UV-Vis measurements at 340 nm. The system was assembled using a rapid dilution protocol.[35] Equimolar amounts of N_{term}-mDHFR, C_{term}-mDHFR (3)-oligonucleotide conjugates and DNA template in 8 M of urea were pre-mixed and diluted into the reaction buffer allowing for protein refolding and DNA hybridization to occur. The progress of the enzymatic reaction was monitored by following the consumption of NADPH in time using UV-Vis measurements at 340 nm. In the absence of template DNA, no catalytic activity was observed; the observed decrease in NADPH absorption coincides with that found for the uncatalyzed NADPH degradation. When including the fully complementary DNA template in the reaction, a rapid consumption of NADPH was observed, demonstrating that the assembled system indeed

is catalytically active (Figure 15).

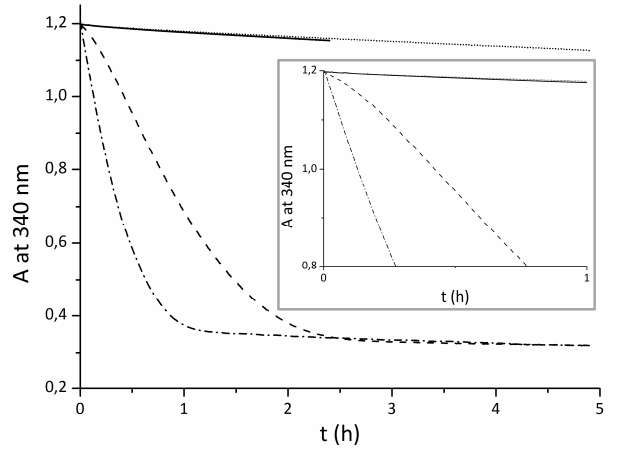


Figure 15. Kinetic curves for the consumption of NADPH in the reduction of dihydrofolate to tetrahydrofolate. Where (—) is the uncatalyzed NADPH degradation, (·····) is the split mDHFR system without DNA template, (— —) is the split mDHFR system with fully complementary template and (—·—) is wild type-mDHFR. Insert depicts the initial stage of the reaction.

A lag phase is observed in the early stages of the reaction. Therefore, in order to allow quantitative comparison of reaction rates, the initial 15 percent of the reaction was not considered. Compared to the wild type mDHFR, the DNA based system is only 2.5 fold slower (Table 1, entries 1 and 3), which suggests that the conformation of the reassembled enzyme closely resembles that of the wild type enzyme.

Table 1. Initial rates for the catalyzed reduction of dihydrofolate to tetrahydrofolate.[a]

Entry	Template	Eq. template	Initial rate ($\cdot 10^{-9}$ M $\cdot$ s^{-1})
1	-[b]	-	71.05 ± 10.09
2	No template	-	0.98 ± 0.66[c]
3	Fully complementary	1	27.90 ± 3.90

[a] All experiments were performed in triplicate using equimolar amounts of protein-DNA conjugates and DNA templates to a final concentration of 0.1 µM, 100 µM dihydrofolate and 100 µM NADPH in Tris buffer (50 mM, pH 7.7, 5 mM $MgCl_2$, 3.3 mM KCl, 10 mM DTT) at 25 °C, unless noted otherwise. Calculated from the slope of the kinetic curves at 340 nm. Corresponds to 10% of the reaction discarding the initial 15%, unless noted otherwise. Errors are calculated from standard deviations. [b] 0.1 µM of full-length mDHFR. [c] Initial 15% of the kinetic curve is discarded.

These results clearly demonstrate that the secondary interactions provided by the DNA template result in the assembly of an active enzyme and induce catalysis of the dihydrofolate reduction.

With the DNA-based split enzyme systems investigated here, a lag phase is observed before catalysis occurs efficiently. A tentative explanation is that, upon dilution into the reaction buffer, some time is required for the hybridization and folding of the system to a catalytically active conformation to occur. However, the lag phase was still observed

when the system was preincubated for 2 hours either in the reaction buffer or in the buffer containing dihydrofolate. Moreover, in these cases a significantly decreased activity was found (Figure 16). Most likely, in addition to the DNA driven reassembly other interactions are required for obtaining the active conformation of the enzyme. Based on earlier work on the homologous *E. coli* enzyme, it is hypothesized that binding of both, dihydrofolate and NADPH is also required for folding in an active conformation.[26]

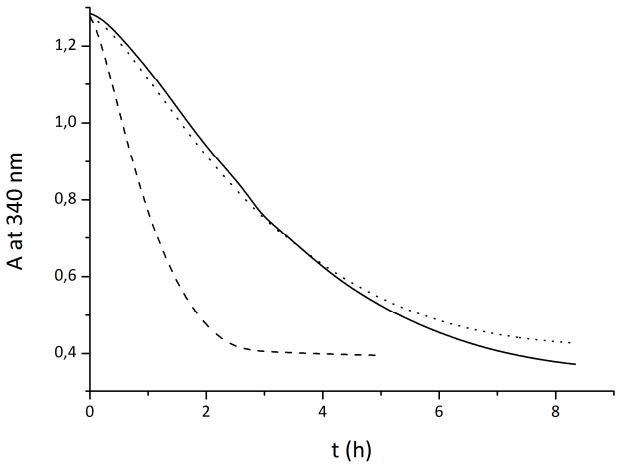


Figure 16. Kinetic curves for the consumption of NADPH in the reduction of dihydrofolate to tetrahydrofolate for equimolar fully complementary systems with preincubation of the system in the reaction buffer (—), in the reaction buffer containing dihydrofolate (….) and without preincubation (---).

4.6. Effect of the template concentration

The catalytic activity was found to be dependent on the concentration of the DNA template (Table 2).

With less than 1 equivalent of template with respect to N_{term}-mDHFR and C_{term}-mDHFR (3) conjugates a lower activity was observed, as expected. Maximum activity was observed when 1 equivalent of template was used. Increasing the concentration of the DNA template to >1 equivalent also results in a progressively lower activity. This is attributed to the fact that with more than equimolar amounts of template present, a mixture of the active reassembled system and templates hybridized with only the N_{term}-mDHFR or the C_{term}-mDHFR (3) conjugate, which are not active, are formed, resulting in an overall decrease of activity. Moreover, experiments in which the refolding of full-length mDHFR were performed in the presence of free DNA showed lower activity than when the DNA was not present indicating that excess of DNA may also inhibit the enzyme. Additional experiments were done in which DNA was added after protein refolding showing no influence in the catalysis. This indicates that the excess of DNA would just affect the results by altering the refolding of the protein and not by blocking the active site once the protein is folded.

Table 2. Initial rates for the catalyzed reduction of dihydrofolate to tetrahydrofolate in the presence of different concentrations of DNA template.[a]

Entry	Eq. template	Initial rate ($\cdot 10^{-9}$ M $\cdot$ s^{-1})
1	1	27.90 ± 3.90
2	0.11	6.14 ± 0.80[b]
3	0.22	9.07 ± 1.56[b]
4	0.34	13.46 ± 2.23
5	0.60	24.74 ± 6.18
6	3	24.05 ± 1.11
7	6	16.02 ± 2.02[b]

[a] All experiments were performed in triplicate using equimolar amounts of protein-DNA conjugates to a final concentration of 0.1 µM with the fully complementary DNA template, 100 µM dihydrofolate and 100 µM NADPH in Tris buffer (50 mM, pH 7.7, 5 mM $MgCl_2$, 3.3 mM KCl, 10 mM DTT) at 25 °C. Calculated from the slope of the kinetic curves at 340 nm. Corresponds to 10% of the reaction discarding the initial 15%. Errors are calculated from standard deviations. [b] Results correspond to the average of two experiments.

4.7. Detection of mismatches

The effect of mismatches in the DNA template on catalytic activity was investigated (Table 3).

Table 3. Initial rates for the catalyzed reduction of dihydrofolate to tetrahydrofolate for DNA template sequences containing different number of mismatches.[a]

Entry	Template	Initial rate ($\cdot$ 10^{-9} M $\cdot$ s^{-1})
1	Fully complementary	27.90 ± 3.90
2	1 mismatch	21.19 ± 1.53
3	3 mismatches	19.85 ± 1.67
4	5 mismatches	13.66 ± 0.94

[a] All experiments were performed in triplicate using equimolar amounts of protein-DNA conjugates and DNA templates to a final concentration of 0.1 µM, 100 µM dihydrofolate and 100 µM NADPH in Tris buffer (50 mM, pH 7.7, 5 mM $MgCl_2$, 3.3 mM KCl, 10 mM DTT) at 25 °C. Calculated from the slope of the kinetic curves at 340 nm. Corresponds to 10% of the reaction discarding the initial 15%. Errors are calculated from standard deviations. Sequence of template: 5'-d(CAAGGTCAGACATGTCAGCGTTCTCACC AGTC) - 3'; residues exchanged are underlined.

Using 1 equivalent of template, a decrease in catalytic activity was found when increasing the number of mismatches. Compared to the full complementary template the activity was reduced by 50 % when 5 mismatches were introduced in the template at positions

12, 14, 16, 20 and 27. The observed activity with 1 and 3 mismatches at positions 16 and 16, 20 and 27 respectively, was found to be similar. This is due to the fact that the three mutations are distributed over the template with 1 and 2 mismatches in the sequences complementary to Oligo 1 and 2, respectively. Hence, the observed decrease in activity is less than would be expected with all 3 mutations in the sequence complementary to oligonucleotide 1 or 2. Combined, these results show that destabilization of the DNA duplex results in a decreased activity. This can be tentatively explained by assuming that a weaker duplex results in more structural flexibility in the protein part, resulting in less of the protein being present in the catalytically active conformation.

4.8. Summary and conclusions

In this chapter, an allosteric system based on a split enzyme covalently tethered to oligonucleotides has been studied. Two different methodologies were investigated for the coupling of the protein fragments to the oligonucleotides, i.e., a maleimide coupling strategy and 1, 3 -dipolar cycloaddition. Although higher conversions were observed in the latter approach, difficulties were encountered in the purification of the conjugates. Therefore, the first approach was selected as the method of choice. The purification step involved two different purification techniques to remove the uncoupled protein and oligonucleotide resulting in highly pure conjugates. Results in the catalysis experiments showed high activity of the system when the fully complementary template was used and lower activities when different concentrations of template or different number of mismatches were incorporated in the sequence.

In conclusion, here we have shown that the catalytic activity of a split enzyme, that is, mDHFR, can be modulated by the concentration and sequence complementarity of a DNA template. This represents a novel and versatile approach to artificial allosteric enzymes. Particularly attractive of the present DNA-based design is that in addition to DNA-controlled catalytic activity, it is envisioned that the concept can be adapted readily for small molecule dependent control over catalytic activity, by inclusion of DNA aptamer structures in the template strand. [36]

4.9. Experimental section

General remarks

3-Maleimidopropionic acid N-hydroxysuccinimide ester was purchased from Alfa Aesar. 6-maleimidohexanoic acid N-hydroxysuccinimide ester was purchased from TCI Europe. Amino modified oligonucleotides were purchased from Biotez Berlin. *E.coli* strains XL1-Blue and Bl21 (DE3) (Stratagene) were used for routine cloning and protein production, respectively. PCR reactions were carried out using a Techne TC-312 apparatus. DNA sequencing was carried out by GATC-Biotech (Berlin, Germany). Primers were synthesized

by Isogen Life Science (De Meern, the Netherlands). Oligonucleotides were purchased from BioTez Berlin-Buch GNbH. Restriction endonucleases and plasmid pTWIN1 were purchased from New England Biolabs. T4 DNA ligase, DNA Gel Extraction Kit and Plasmid Puriying Kit were purchased from Roche. *Pfu Turbo* polymerase was purchased from Stratagene. Plasmid pQE-30Xa was purchased from Qiagen. Plasmid pET-17b was purchased from Novagen. Commercially available plasmid pQE-16 was kindly provided by Prof. Dr. S.W. Michnick (University of Montreal, Canada). DNA manipulations were done by standard procedures.[37]

Ni-NTA agarose was purchased from Qiagen. HiTrap QFF column was purchased from GE Healthcare. Reversed phase-HPLC analysis were performed on a Shimadzu LC-10AD VP, Waters Xterra MS C18 column (3.0 x 150 mm, particle size 3.5 µm) using a gradient of CH_3CN/Triethylammonium acetate (TEAA) buffer 50 mM pH 7; gradient: 05/95 0 to 10 min, to 35/65 at 60 min, to 70/30 at 65 min. Flow: 0.5 mL/min. Centrifugation was done using a Beckman Coulter Avanti J-E centrifuge. Size exclusion chromatography was done using a Superdex 75 HR 10/30 column from Pharmacia. MALDI-TOF measurements were done on a Voyager-DE Pro apparatus. (Matrix for oligonucleotide samples: 20 µL of a solution of 2, 4, 6- trihydroxyacetophenone 0.5 M in ethanol + 10 µL of a solution of ammonium citrate dibasic 0.1 M in Milli Q water + 2 µL sample solution in Milli Q water; matrix for protein samples: 1 mg/mL of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile, 0.1% trifluoroacetic acid, 1-10 pmol of protein sample). Concentration determinations were done using a Nanodrop ND-1000 from Thermo Scientific using the calculated extinction coefficients ε_{260nm} = 163840 $M^{-1} \cdot cm^{-1}$ for Oligo 1 and ε_{260nm} = 179210 $M^{-1} \cdot cm^{-1}$ for Oligo 2. The same values were used for the corresponding mDHFR-oligo conjugates, since the absorption of the protein fragments at 260 nm is neglible compared to the oligonucleotide absorption. UV/Vis measurements were recorded on a JASCO V-560/V-570 UV/Vis Spectrometer at 25 °C. mDHFR assays were conducted in quartz cuvettes with a 1 cm path length. Äkta Purifier 900 (Amersham Biosciences) was used for Fast Protein Liquid Chromatography (FPLC).

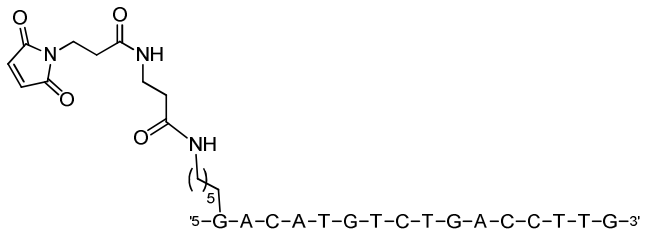
3-Azidopropanoic acid N-hydroxysuccinimide ester[38] and 2-iodo-N-(prop-2-yn-1-yl)acetamide[39] were synthesized according to published procedures.

Synthesis of maleimido-DNA conjugates, representative procedure:

182 µL of a stock solution of amino modified-oligonucleotide (200 µM in H_2O) was mixed with 236 µL of phosphate buffer (200 mM, pH 7.2). To this solution, 60 µL of a stock solution of 3-maleimidopropionic acid N-hydroxysuccinimide ester (30 mg/mL in N, N-dimethylformamide) was added. The mixture was gently mixed and allowed stand for 2 hours. Longer incubation times resulted in hydrolysis of the maleimide ring. The conjugate was purified by size exclusion chromatography (SephadexTM G-25 DNA Grade,

TEAA 50 mM pH 7) and lyophilized. The products were analyzed by reversed phase-HPLC and MALDI-TOF.

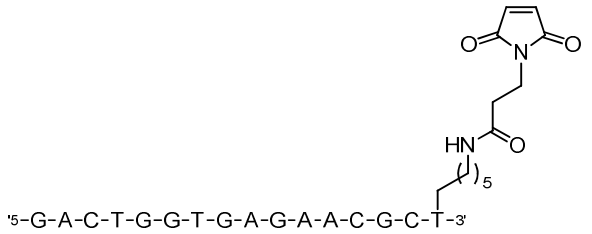
Oligo 1:



RP-HPLC: retention time = 32 min

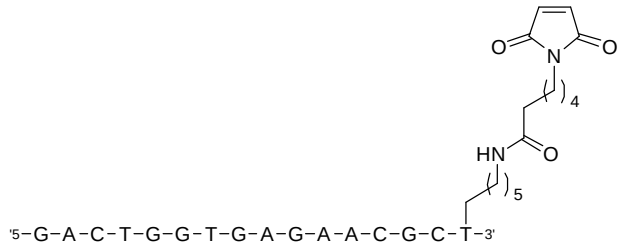
MALDI-TOF m/z 5300 (calcd. 5290)

Oligo 2:



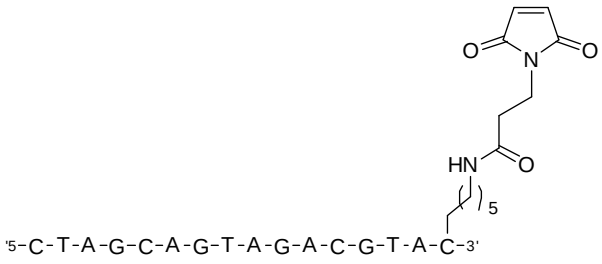
RP-HPLC: retention time = 39 min

MALDI-TOF m/z 5275 (calcd. 5276)



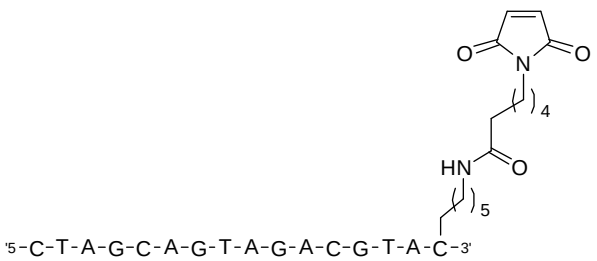
RP-HPLC: retention time = 39 min

MALDI-TOF m/z 5315 (calcd. 5319)



RP-HPLC: retention time = 29 min

MALDI-TOF m/z 5215 (calcd. 5207)

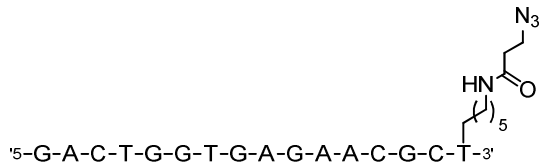


RP-HPLC: retention time = 39 min

MALDI-TOF m/z 5260 (calcd. 5263)

Synthesis of azide-containing DNA conjugates:

182 µL of a stock solution of amino modified-oligonucleotide (200 µM in H_2O) was mixed with 236 µL of Phosphate buffer (200 mM, pH 7.2). To this solution, 60 µL of a stock solution of 3-azidopropanoic acid *N*-hydroxysuccinimide ester (30 mg/mL in *N, N*-dimethylformamide) was added. The mixture was shaken and allowed to stand for 2 hours. The conjugate was purified by size exclusion chromatography (SephadexTM G-25 DNA Grade, TEAA 50 mM pH 7) and lyophilized. The product was analyzed by reversed phase-HPLC and MALDI-TOF.



RP-HPLC: retention time = 35 min

MALDI-TOF m/z 5213 (calcd. 5225)

Construction of plasmid pTWINXa:

The pTWINXa plasmid was derived from commercially available plasmids pTWIN1 and pQE-30Xa.

The intein part of the pTWIN1 vector was substituted by the multiple cloning site (MCS) of pQE-30Xa, including the 6x His-tag and factor Xa recognition site. The MCS including the 6x His-tag and factor Xa recognition site was amplified by polymerase chain reaction (PCR) using pQE-30Xa as template. PCR primers were as following; primer 1: 5'-TACTACATATGAGAGGATCGCATC-3' (including *Nde*I restriction site, underlined), primer 2: 5'-GCTCAGCTAATTAAGCTT-3'. PCR cycles were as following: initial denaturation at 94 ^{o}C for 5 min. Denaturation at 94 ^{o}C for 1 min, annealing at 40 ^{o}C for 45 sec., extension at 72 ^{o}C for 25 sec, for 30 cycles. Final extension at 72 ^{o}C for 5 min. The obtained PCR product was digested with *Nde*I and *Pst*I, and inserted between the same sites of the expression vector pTWIN1.

Construction of expression plasmid pTWINXa-mDHFR:

The mDHFR gene was amplified by PCR using pQE-16 as template. PCR primers were as following; primer 1: 5'-GTTCGACCATTGAACTCGAT-3', primer 2: 5'-TACTACCTGCAGTTAAT CTTTCTTCTCGTAGACTTCAA-3' (including *Pst*I restriction site, underlined). PCR cycles were as following: initial denaturation at 94 ^{o}C for 5 min. Denaturation at 94 ^{o}C for 1 min, annealing at 42 ^{o}C for 45 sec., extension at 72 ^{o}C for 4 min, for 30 cycles. Final extension at 72 ^{o}C for 5 min. The obtained PCR product was digested with *Pst*I and pTWINXa was digested with *Stu*I (blunt-end cutter) and *Pst*I and both were ligated together using T4 ligase.

Construction of expression plasmid pTWINXa-N_{term}-mDHFR:

The N_{term}-mDHFR gene was amplified by PCR using pQE-16 as template. PCR primers were as following; primer 1: 5'-GTTCGACCATTGAACTCGAT-3', primer 2: 5'-ACTACCTGCAGTTAG CACAATTCCGGTTGTTCAATAA-3' (including DNA sequence for the incorporation of a cysteine, underlined). PCR cycles were as following: initial denaturation at 94 ^{o}C for 5 min. Denaturation at 94 ^{o}C for 1 min, annealing at 50 ^{o}C for 45 sec., extension at 72 ^{o}C for 25 sec., for 15 cycles. Followed by denaturation at 94 ^{o}C for 1 min, annealing at 48 ^{o}C for 45 sec., extension at 72 ^{o}C for 25 sec., for 15 cycles. Final extension at 72 ^{o}C for 5 min. The obtained PCR product was digested with *Pst*I and pTWINXa was digested with *Stu*I (blunt-end cutter) and *Pst*I and both were ligated together using T4 ligase.

Construction of expression plasmid C_{term}-mDHFR (1).

The Ligation Independent Cloning (LIC) technique was used to construct C_{term}-mDHFR (1). The LIC vector, containing an N-terminal 10 His-tag followed by a TEV protease cleavage site, was kindly provided by Dr. E. Geertsma (University of Groningen). The generation of the LIC cassette was done by PCR using the pQE-16 vector as a source for the mDHFR gene, with the following primers: Forward primer 5'- ATGGTGAGAATTTATATTTTCAA TGTTTGGCAAGTAAAGTAGACATGGTTTGG-3' (TEV recognition site underlined), reverse primer 5'-TGGGAGGGTGGGATTTTCATTA TTTCTTCTCGTAGACTTCAAACTTAT-3'. The PCR cycling conditions were as following: initial denaturation at 94 ^{o}C for 5 min. 30 cycles with 1 min. denaturation at 94 ^{o}C, 45 sec. annealing at 45 ^{o}C and 1.5 min. extension at 72 ^{o}C. Final extension at 72 ^{o}C for 5 min. The cassette was purified from a 1.2% TEA DNA gel, using the standard gel extraction kit from Qiagen. The LIC vector was digested with SwaI, incubated at 25 ^{o}C for 3h, purified again by gel extraction and eluted with 25 µL TIoWE buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA). The vector was treated with T4 DNA polymerase in a total volume of 15µL, containing 200ng vector, 2.5 mM dCTP, 0.5U of T4 DNA polymerase (Roche) and the appropriate buffer. The mixture was incubated at 25 ^{o}C for 30 min, subsequently, the T4 DNA polymerase was inactivated by incubating at 75 ^{o}C for 20 min, and cooled to 4 ^{o}C. The cassette was treated with T4 DNA polymerase under

the same conditions, with the following exceptions: 15 ng of cassette and 2.5 mM dGTP instead of 2.5 mM dCTP. The LIC ready vector and cassette were mix in a volume ratio of 1:3 vector/cassette, and incubated at RT for 5 min. 3 µL of the mixture was transformed to $CaCl_2$ competent *E.coli* TOP10 cells using the standard transformation techniques and cells were plated for selection of ampicillin resistant clones. Single transformant colonies were grown overnight at 37 ^{o}C in 5 mL LB/Amp.

Construction of expression plasmid pTWINXa-C_{term}-mDHFR (2):

The C_{term}-mDHFR (1) gene was amplified by PCR using pQE-16 as template. PCR primers were as following; primer 1: 5'-TACTACATATGGGCTGCGCAAGTAAAGTAGACA-3' (including *Nde*I restriction site, underlined), primer 2: 5'-TACTACTGCAGCTCAGCTAATTAA GCTT-3' (including *Pst*I restriction site, underlined). PCR cycles were as following: initial denaturation at 94 ^{o}C for 5 min. Denaturation at 94 ^{o}C for 1 min, annealing at 42 ^{o}C for 45 sec., extension at 72 ^{o}C for 50 sec., for 30 cycles. Final extension at 72 ^{o}C for 5 min. The obtained PCR product was digested with *Nde*I and *Pst*I, and inserted between the same sites of the vector pTWIN1Xa.

Construction of expression plasmid pTWINXa-C_{term}-mDHFR (3):

The C_{term}-mDHFR (3) gene was amplified by PCR using pTWINXa-C_{term}-mDHFR (2) as template, introducing a glycine/serine linker. PCR primers were as following; primer 1: 5'-TACTACCATATG*GGCTGCGGCGGCAGTGGCGGC*GCAAGTAAAGTAGACATGGTTTGG- 3' (including *Nde*I restriction site, underlined; Glycine/serine linker in *Italic*), primer 2: 5'-GTAGTACTGCAGTTAGTGATGGTGATGGTGATGAG – 3' (including *Pst*I restriction site, underlined). PCR cycles were as following: initial denaturation at 94 ^{o}C for 5 min. Denaturation at 94 ^{o}C for 1 min, annealing at 45^{o}C for 45 sec., extension at 72 ^{o}C for 90 sec., for 30 cycles. Final extension at 72 ^{o}C for 5 min. The obtained PCR product was digested with *Nde*I and *Pst*I, and inserted between the same sites of the expression vector pET17b.

Expression and purification of mDHFR fragments:

Protein expression plasmids of the individual N_{term}-mDHFR, C_{term}-mDHFR and mDHFR constructs were transformed into *E. coli* BL21 (DE3) (C_{term}-mDHFR (1) construct was transformed into *E. coli* TOP10) and a single colony was inoculated into a starter culture of 5 mL of fresh LB medium containing 100 µg/mL of ampicillin. 1 mL of starter culture was used to inoculate 200 mL of fresh LB medium containing 100 µg/mL of ampicillin. When the culture reached the mid-log phase (optical density at 600 nm around 0.6–0.8) isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.2% of L-arabinose was used for C_{term}-mDHFR (1)) was added to induce the expression of target protein at final concentration of 1 mM. Expressions were done at 37 ^{o}C for N_{term}-mDHFR and mDHFR and at 30 ^{o}C for C_{term}-

mDHFR overnight. Cells were harvested by centrifugation (6000 rpm, JA-10, 25 min, 4 °C), followed by resuspension in 4 mL of 100 mM NaH_2PO_4, pH 8.0, 100 mM Tris-HCl, 8 M urea. The lysate was centrifuged (10000 rpm, JA-17, 25 min, r.t.). The supernatant was equilibrated with 2 mL of pre-equilibrated slurry of Ni-NTA (50% Ni-NTA in 20% ethanol) for 1 h (mixed at 200 rpm on a rotary shaker) at room temperature. The column was washed with 2 fractions of 4 mL of 100 mM NaH_2PO_4, pH 6.3, 100 mM Tris-HCl, 8 M urea, 10 mM imidazole, subsequently, eluted with 5 fractions of 0.5 mL of 100 mM NaH_2PO_4, pH 4.0, 100 mM Tris-HCl, 8 M urea. Fractions were analyzed on a 12% polyacrylamide SDS-Tris Tricine gel followed by Coumassie staining. The concentration of the protein fragments was determined by using the following calculated extinction efficients: N_{term}-mDHFR ε_{280} = 12490 $M^{-1} \cdot cm^{-1}$, C_{term}-mDHFR (1) ε_{280} = 14440 $M^{-1} \cdot cm^{-1}$, C_{term}-mDHFR ε_{280} = 12950 $M^{-1} \cdot cm^{-1}$. The literature[40] value for mDHFR is ε_{280} = 74600 $M^{-1} \cdot cm^{-1}$ (Calculations were done by Protparam on the Expasy server).[41]

Expression yields were: 11 mg/L for N_{term}-mDHFR, 30 mg/L for C_{term}-mDHFR (1), 29 mg/L for C_{term}-mDHFR (2) and 5 mg/L for C_{term}-mDHFR (3).

MS (ESI) N_{term}-mDHFR 14345 Da (calcd. 14341 Da); C_{term}-mDHFR (1) 10694 Da (calcd. 10824 Da, calcd. [M-Met] 10692 Da), C_{term}-mDHFR (2) 11009 Da (calcd. 11140 Da, calcd. [M-Met] 11008 Da)

General procedure for the synthesis of protein-DNA conjugates via maleimide coupling:

A volume of the mDHFR fragment solution corresponding to 93 nmol of protein was brought to pH 8 using NaOH 6 M solution and incubated with dithiothreitol (DTT), 0.1 M solution for 1 h at room temperature. To this solution, 1 mL of 25% trifluoroacetic acid (TFA) was added and the mixture was incubated on ice for 1 h. The mixture was centrifuged at maximum speed for 5 min and the pellet was washed with water several times. The pellet was redissolved in 8 M urea 100 mM CHES, pH 9.2 to a final concentration of 80 µM. The solution was immediately transferred to the freeze-dried maleimide modified oligonucleotide (1:1 ratio of functionalized oligonucleotide/mDHFR fragment). The mixture was rotated by a rotator (100 rpm) at 40 °C for 16 h. The extent of coupling was analyzed on a 12% polyacrylamide SDS-Tris Tricine gel, following Coumassie staining.

General procedure for the purification of the protein-oligonucleotide conjugates:

- Purification from uncoupled mDHFR fragments: The conjugates were purified by anion-exchange chromatography on a HiTrap QFF column by a gradient of NaCl concentration from 0 to 1 M in 5 min with a flow of 0.5 mL $\cdot$ min^{-1} in a 100 mM Tris-HCl, pH 7.5, 8 M urea buffer. Elution fractions were analyzed by SDS-PAGE.

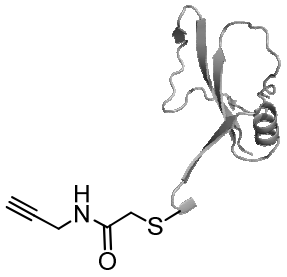
- Purification from uncoupled oligonucleotide: The conjugates were purified by Ni-NTA agarose column. The pooled fractions from the anion exchange column containing the conjugate were equilibrated with 100 µL of slurry Ni-NTA (50% Ni-NTA in 20% Ethanol) for 0.5 h. The column was pre-equilibrated with 100 mM NaH_2PO_4, pH 8.0, 100 mM Tris-HCl, 8 M urea. Subsequently, the column was washed with 2 fractions of 500 µL of 100 mM NaH_2PO_4, pH 8.0, 100 mM Tris-HCl, 8 M urea and eluted with 8 fractions of 50 µL of 100 mM NaH_2PO_4, pH 4.0, 100 mM Tris-HCl, 8 M urea.

Characterization of protein-oligonucleotide conjugates by size exclusion chromatography:

The conjugates were analyzed by analytical size exclusion chromatography using a Superdex 75 HR 10/30 column, pre-equilibrated with 100 mM Tris-HCl, pH 7.5, 8 M urea buffer. The elution volume of the conjugates is at 9 mL.

Alkyne modification of C_{term}-mDHFR (2):

A volume of the C_{term}-mDHFR (2) solution in 100 mM NaH_2PO_4, pH 4, 100 mM Tris-HCl, 8 M urea, as obtained from the purification step on the Ni-NTA column, corresponding to 106 nmol was brought to pH 8 using NaOH 6 M solution and incubated in dithiothreitol (DTT) 0.1 M solution for 1 hour at room temperature. To this solution, 1 mL of 25% trifluoroacetic acid (TFA) was added and the mixture was incubated on ice for 1 hour. The sample was centrifuged at maximum speed for 5 minutes, the pellet rinsed several times with Milli Q water and stored under nitrogen atmosphere. 990 µL of deoxygenated buffer (500 mM Tris-HCl, pH 8.5, 2 mM EDTA, 8 M urea) were added to the protein pellet followed by 29.6 µL of 2-iodo-N-(prop-2-yn-1-yl)acetamide stock solution (89.7 mM in DMF) under nitrogen. The reaction mixture was shaken overnight. The protein fragment was precipitated using 4 mL of 25% TFA solution for 1 h in ice. The sample was centrifuged at maximum speed for 5 min., decanted and rinsed with Milli Q water. The protein pellet was used in the coupling to azide-modified oligonucleotides by redissolving it in the corresponding buffer.



MALDI-TOF m/z 10776 (calcd. 10789)

ESI m/z 10790

General procedure for the synthesis of protein-DNA conjugates via 1, 3 dipolar cycloaddition:

A Cu (I) stock solution was prepared by mixing 15 µL of a 1.2 µM $CuSO_4$ solution and 15 µL of a 1,9 µM TCEP solution in deoxygenated Milli Q water and stored under nitrogen atmosphere. 134 nmol of azide-DNA conjugate was dissolved in 492 µL of deoxygenated phosphate buffer (100 mM, pH 7.9, 8 M urea). 32 µL of Cu (I) stock solution and 12 µL of a 1.1 mM alkyne modified protein solution in deoxygenated buffer were added. The vial was sealed and mixed at 37 ^{o}C for two days. The extent of coupling was analyzed on a 12% polyacrylamide SDS-Tris Tricine gel, followed by Coumassie staining.

Protein sequences:

Full length m-DHFR

MRGSHHHHHHGSGSGSGIEGRVRPLNSIVAVSQNMGIGKNGDLPWPPLRNEFKYFQRMT
TTSSVEGKQNLVIMGRKTWFSIPEKNRPLKDRINIVLSRELKEPPRGAHFLAKSLDDALRLIEQP
ELASKVDMVWIVGGSSVYQEAMNQPGHLRLFVTRIMQEFESDTFFPEIDLGKYKLLPEYPGV
LSEVQEEKGIKYKFEVYEKKD

N_{term}-mDHFR

MRGSHHHHHHGSGSGSGIEGRVRPLNSIVAVSQNMGIGKNGDLPWPPLRNEFKYFQRMT
TTSSVEGKQNLVIMGRKTWFSIPEKNRPLKDRINIVLSRELKEPPRGAHFLAKSLDDALRLIEQP
ELC

C_{term}-mDHFR (1)

MHHHHHHHHH HGENLYFQCL ASKVDMVWIV GGSSVYQEAM NQPGHLRLFV
TRIMQEFESD

TFFPEIDLGK YKLLPEYPGV LSEVQEEKGI KYKFEVYEKK

C_{term}-mDHFR (2)

GCASKVDMVWIVGGSSVYQEAMNQPGHLRLFVTRIMQEFESDTFFPEIDLGKYKLLPEYPGV
LSEVQEE KGIKYKFEVYEKKGSRSHHHHHH

C_{term}-mDHFR (3) (linker appears in bold)

GC**GGSGG**ASKVDMVWIVGGSSVYQEAMNQPGHLRLFVTRIMQEFESDTFFPEIDLGKYKLL
PEYPGVLSEVQEEKGIKYKFEVYEKKGSRSHHHHHH

4.10. References

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